Human N-acetylgalactosamine-4-sulphatase biosynthesis and maturation in normal, Maroteaux–Lamy and multiple-sulphatase-deficient fibroblasts

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The biosynthesis and maturation of N-acetylgalactosamine-4-sulphatase (4-sulphatase) was studied in normal fibroblasts and in fibroblasts from patients with either mucopolysaccharidosis type VI (MPS VI; Maroteaux–Lamy syndrome) or multiple sulphatase deficiency (MSD). Fibroblasts were incubated in culture medium containing [³H]leucine or [³⁵S]methionine, and radiolabelled 4-sulphatase was isolated by immunoaffinity chromatography using 4-sulphatase-specific monoclonal antibodies. In normal fibroblasts a precursor of 66 kDa, detected intracellularly after 3 h and in NH₄Cl-induced secretions, was processed intracellularly, within an additional 3 h, to a polypeptide of 57 kDa composed of disulphide-linked polypeptides of 43 kDa and 8 kDa. All fibroblast lines obtained from MPS VI patients, exhibiting clinical characteristics ranging from no clearly recognized symptoms to the severe classical phenotype, incorporated radioactivity into immune-purified 4-sulphatase at a rate less than 10 % of that seen in normal fibroblasts. Maturation of the residual 4-sulphatase showed, variously, features which may be indicative of delayed intracellular transport, decreased intracellular stability, failure of lysosomal targetting or resistance to enzyme processing. Although some features of the residual enzyme synthesis and maturation were consistent with the patient's clinical phenotype, this was infrequent. The maturation of 4-sulphatase in fibroblasts from MSD patients was indistinguishable from that in normal fibroblasts, and the half-life of 4-sulphatase in these fibroblasts, determined after a 24 h pulse and prolonged chase, was only slightly less than that in normal fibroblasts.

INTRODUCTION

Mucopolysaccharidosis type VI (MPS VI) or Maroteaux–Lamy syndrome is an autosomal recessive disorder of glycosaminoglycan catabolism which results from a deficiency of the lysosomal enzyme N-acetylgalactosamine-4-sulphatase (4-sulphatase; EC 3.1.6.1) and leads to the storage and excretion of large amounts of dermatan sulphate. MPS VI patients present with a variety of clinical phenotypes, the severe form resulting in hepatosplenomegaly, corneal opacity, skeletal malformation and growth retardation, but with normal, or near normal, neuronal development (McKusick & Neufeld, 1983). A deficiency of 4-sulphatase activity is not unique to MPS VI. Sulphated mucopolysaccharides also accumulate in a rare genetic disorder, termed multiple sulphatase deficiency (MSD), which is characterized by the partial deficiency of all known lysosomal sulphatases as well as the microsomal steroid sulphatase (Kolodny & Moser, 1983; Shapiro, 1983).

Lysosomal enzymes are synthesized as large-molecular-mass precursors that are proteolytically processed in acid compartments to mature forms of lower molecular mass (Hasilik & Neufeld, 1980; Gieselmann et al., 1983; Oude Elferink et al., 1986). The synthesis and maturation properties of 4-sulphatase have previously been analysed in normal and MPS VI fibroblasts (Steckel et al., 1983). That study utilized a polyclonal antibody directed against 4-sulphatase to reveal polypeptides of 64 kDa, 57 kDa, 47 kDa, 40 kDa, 31 kDa and 11 kDa, which were proposed to be involved in the biosynthesis and processing of 4-sulphatase. In the present study, the efficient recovery of 4-sulphatase by monoclonal-antibody affinity chromatography and the subsequent SDS/polyacrylamide-gel electrophoresis of the

protein suggest that the 40 kDa and 30 kDa polypeptides detected by Steckel et al. (1983) are not related to 4-sulphatase. This has important implications in the interpretation of 4-sulphatase biosynthesis and processing, particularly in fibroblasts from MPS VI and MSD patients.

The present paper describes the biosynthesis and processing of 4-sulphatase in fibroblast cell lines derived from normal subjects, MPS VI patients and MSD patients with the aim of describing mutations in 4-sulphatase synthesis and investigating the relationship between the expression of 4-sulphatase processing intermediates and the clinical phenotype observed in MPS VI patients. Defining the precise nature of the defect in MPS VI patients is directly relevant to the subsequent treatment of patients, for example via bone-marrow transplantation, enzyme-replacement therapy or gene-replacement therapy.

MATERIALS AND METHODS

Materials

L-[3,4,5-³H(n)]Leucine (sp. radioactivity 144–153 Ci/mmol), L-[³⁵S]methionine (sp. radioactivity 1215–1478 Ci/mmol), Hyperfilm-MP and a mixture of [¹⁴C]methylated molecular-mass standards (myosin, 200 kDa; phosphorylase b, 92.5 kDa; BSA, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; lysozyme, 14.4 kDa) were purchased from Amersham International (Amersham, Bucks., U.K.). Affi-Gel 10 was purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.) and used according to the manufacturers' recommendations. Dulbecco's modified phosphate-buffered saline was purchased from Commonwealth Serum Laboratories (Melbourne, Vic., Australia).

Nonidet P-40 and proteinase inhibitors were purchased from Sigma (St. Louis, MO, U.S.A.). DNAase was purchased from Boehringer-Mannheim (Sydney, N.S.W., Australia). Eagle's modified minimum essential medium, penicillin, streptomycin and glutamine were purchased from Flow Laboratories (Sydney, N.S.W., Australia), and foetal-calf serum was from Gibco (Glen Waverley, Vic., Australia).

Methods

Cell culture. Human diploid fibroblasts were established from skin biopsies submitted to this hospital for diagnosis (Hopwood et al., 1982). Cell lines were maintained at 37 °C in air/CO_2 (19:1) in Eagle's modified minimum essential medium, supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), glutamine (300 μg/ml) and 10% (v/v) foetal-calf serum. Fibroblast cultures were not used beyond 15 passages.

The MSD cell lines GM 3245 and GM 4681 were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ, U.S.A.), and cell line N.B. was referred to us by Professor D. Patrick, Institute of Child Health, University of London, U.K. The two cell-bank repository lines GM 3245 and GM 4681 were classified as multiple-sulphatase-deficient fibroblasts Group II (residual sulphatase activity up to 90%) as described by Steckel et al. (1985), whereas cell line N.B. was from a patient classified as clinically moderate.

Incorporation of radiolabelled amino acids into 4-sulphatase. At confluency, fibroblast cells were washed once in Dulbecco's phosphate-buffered saline, pH 7.4, supplemented with 1% foetal-calf serum, and then incubated for 2 h in 5.0 ml of methionine-free or leucine-free medium containing 10% foetal-calf serum. Cells were then labelled for specified time periods in 5.0 ml of cell-culture medium containing either [^{35}S]methionine (0.15 mCi/75 cm^2 flask) or [^{3}H]leucine (0.3 mCi/75 cm^2 flask). The labelled cells were either harvested immediately or chased for the times specified, in 5.0 ml of cell-culture medium containing 10% foetal-calf serum and either 15 mM-methionine or 53 mM-leucine. In some instances, cells were cultured as above but with culture medium supplemented with 10 mM-NH_4Cl.

After labelling, culture medium (5.0 ml) was precipitated with 2.5 g of $(NH_4)_2SO_4$ and dialysed against water as described by Hasilik & Neufeld (1980). Cells were harvested by trypsin digestion as described by Conary et al. (1988). The cells were then suspended in 0.5 ml of buffer containing 1% (v/v) Triton X-100, 0.5 mg of sodium deoxycholate/ml, 0.2 mg of SDS/ml, 5 mg of BSA/ml, 1 mM-$MgCl_2$, 1 mM-phenylmethanesulphonyl fluoride, 5 mM-iodoacetamide, 0.020 mg of DNAase/ml, 10 mM-EDTA, 10 mM-ATP, 0.25 M-NaCl and 10 mM-Tris/HCl, pH 7.4 (Buffer A). Cell suspensions were then lysed by freeze-thawing six times, and nuclear material was removed by precipitation with protamine sulphate as described by Hasilik & Neufeld (1980). A solution containing a mixture of the proteinase inhibitors antipain, aprotinin, chymostatin, leupeptin and pepstatin was then added to cell and medium supernatants to give a final concentration of 10 μg of each inhibitor/ml, and supernatants were used directly for immunoadsorption.

Monoclonal antibodies. Monoclonal antibodies designated 4-S 4.1 and 4-S 5.2 raised against 4-sulphatase were characterized as specific for this lysosomal enzyme (Gibson et al., 1987; Brooks et al., 1990). The monoclonal antibodies (2 mg) were covalently linked to Affi-Gel 10 slurry (1.0 ml), and are hereafter referred to as 4-S 4.1 Affi-Gel or 4-S 5.2 Affi-Gel. Affi-Gel 10 was blocked by incubation in 0.02 M-Tris/HCl, pH 7.0, as a control to monitor non-specific binding of radiolabelled polypeptides to the Affi-Gel 10 matrix, and is hereafter referred to as Tris-blocked Affi-Gel.

Immunoadsorption. Purified carrier 4-sulphatase isolated from human lung or liver (Gibson et al., 1987) was added to cell extracts and culture medium at a minimum concentration of 0.4 μg/ml before application to a 0.5 ml column of 4-S 4.1 or 4-S 5.2 Affi-Gel. The columns were incubated overnight at 4 °C and then subjected to an extensive washing regime as originally described by Stein et al. (1987), but modified as outlined below, with successive 1.0 ml washes of: Wash 1, Buffer A; Wash 2, Buffer A, without proteinase inhibitors, $MgCl_2$, EDTA, ATP and DNAase; Wash 3, Buffer A with 2 M-KCl substituted for SDS; Wash 4, 0.6 M-NaCl/0.1% (w/v) SDS/0.05% (v/v) Nonidet P-40/10 mM-Tris/HCl, pH 8.6; and Wash 5, repeated washing with 14 mM-NaCl/1 mM-Tris/HCl, pH 7.4, until radioactivity approached background levels; generally 10–12 1.0 ml volumes were required.

Radiolabelled enzyme and carrier enzyme were then eluted from the immunoaffinity columns by a series of 1.0 ml volumes of 2.0 M-NaCl/0.05 M-sodium citrate, pH 4.0, followed by 4.0 M-NaCl/0.05 M-sodium citrate, pH 3.5. The columns were re-equilibrated with 0.25 M-NaCl/0.02 M-Tris/HCl, pH 7.4. In some experiments the 4-sulphatase obtained in the flow-through from step 1 was re-applied to the column before commencement of the washing and elution regime as outlined above.

SDS/polyacrylamide-gel electrophoresis and fluorography. The purified radiolabelled 4-sulphatase and carrier 4-sulphatase were precipitated by a deoxycholate/tricholoroacetic acid co-precipitation method as described by Mahuran et al. (1983), with the modification that 1% (v/v) triethylamine was included in the acetone washes. Discontinuous gels (12.0% acrylamide unless specified otherwise) were run as described by Laemmli (1970). The unstained gels were fixed overnight in 40% (v/v) methanol/10% (v/v) acetic acid and impregnated with di-phenyloxazole as described by Bonner & Laskey (1974), and dried gels were exposed to Hyperfilm-MP (Laskey & Mills, 1975). Radioactivity in specific bands was quantified from the radioactivity applied, together with determination of relative band intensity.

4-Sulphatase activity. 4-Sulphatase activity recovered from the various steps of the immunopurification process was determined with 4-methylumbelliferyl sulphate substrate as described by Gibson et al. (1987). 4-Sulphatase activities in cultured fibroblasts documented in Table 1 were determined by using a radiolabelled trisaccharide substrate as described by Hopwood et al. (1986).

RESULTS

Recovery of 4-sulphatase after immunoaffinity chromatography

Under the conditions developed, approx. 80% of the carrier 4-sulphatase activity bound to the 4-S 4.1 Affi-Gel. Of this, approx. 30% was eluted gradually during the wash procedure (Fig. 1a). The 10–20% that consistently flowed straight through the column during the sample-application step could be combined with the 4-sulphatase from the Buffer A wash (step 1) and re-applied to the column. As this bound with an efficiency similar to the initial application, a recovery of carrier 4-sulphatase, in the final 2.0 M-NaCl low-pH elution, of 55–68% was regularly achieved. The extensive washing procedure used, particularly repeated washing at step 5, was essential to achieve removal of radioactive contaminants (Fig. 1b). A less vigorous washing protocol produced a variable and unacceptably high level of radiolabelled contaminants in the eluted 4-sulphatase (results not shown). The inclusion of ATP and EDTA, as reported by Peltz et al. (1987), decreased contamination with the abundant cytoplasmic proteins actin (43 kDa) and myosin (200 kDa).
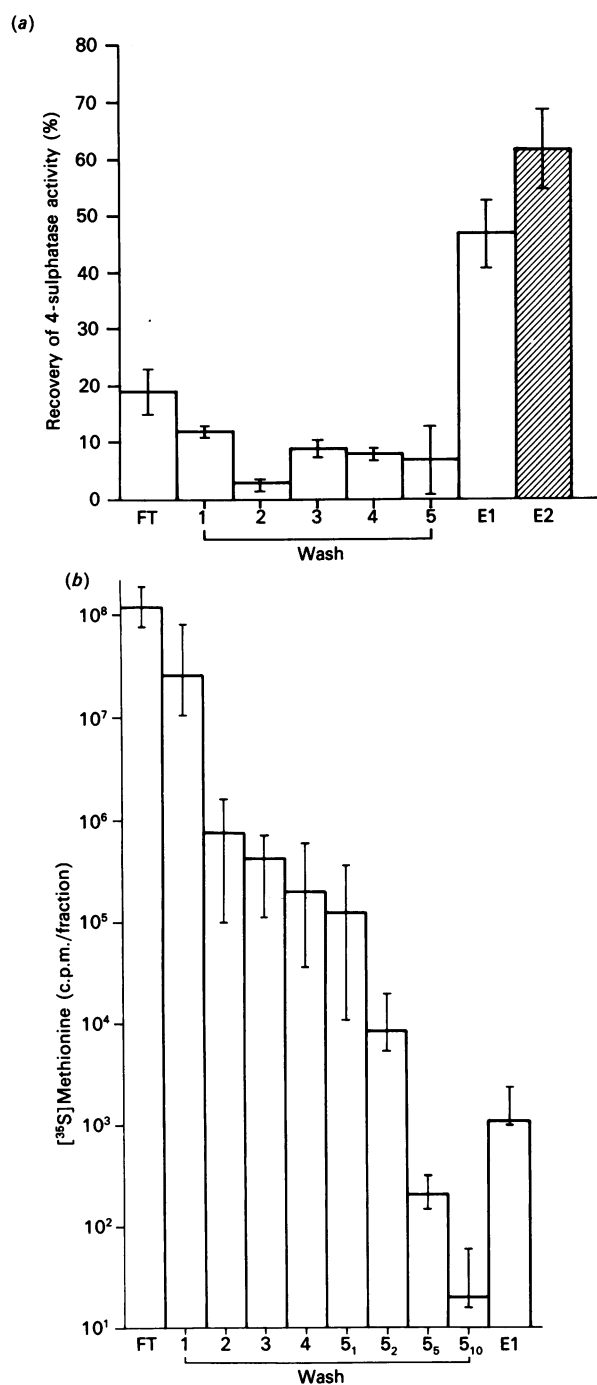
Fig. 1. Recovery of 4-sulphatase after immunopurification

Normal fibroblasts were incubated for 24 h in the presence of [^{35}S]methionine, and 4-sulphatase was isolated by immunopurifications on a 4-S 4.1 Affi-Gel column after adjusting to at least 0.4 μg/ml with purified carrier 4-sulphatase. The column was then subjected to a series of washes before elution of 4-sulphatase with 2.0 M-NaCl/0.05 M-sodium citrate, pH 4.0. The bars indicate the range obtained for five different immunoadsorptions. (*a*) Recovery of carrier 4-sulphatase activity at each wash step and elution expressed as a percentage of applied carrier 4-sulphatase. FT represents the flow-through from the initial application of cell extract. Washes 1–5 are those described in the Materials and methods section. E1 represents the pooled fractions eluted with 2.0 M-NaCl/Tris/HCl, pH 4.0. E2 represents the pooled fractions eluted with 2.0 M-NaCl/Tris/HCl, pH 4.0, after the FT had been re-applied to the column. (*b*) Radioactivity (c.p.m. of [^{35}S]methionine) recovered at each wash step and final elution (E1). 5_1, 5_2, 5_5 and 5_{10} represent washes 1, 2, 5 and 10 of those in step 5 using 14 mM-NaCl/1 mM-Tris/HCl, pH 7.4.

Synthesis and maturation of 4-sulphatase by normal fibroblasts

4-Sulphatase in lysates and the culture medium obtained from fibroblasts radiolabelled for 24 h migrated as a 57 kDa polypeptide on SDS/polyacrylamide-gel electrophoresis. Electrophoresis under reducing conditions showed this to be composed of polypeptides of 43 kDa and 8 kDa (Fig. 2*b*). The 8 kDa polypeptide appeared as a faint band on gels and may not be visible in some photographic reproductions. The molecular mass of this polypeptide, previously referred to as 13 kDa (Gibson *et al.*, 1987), was re-determined by electrophoresis of both purified human lung and kidney 4-sulphatase on a 15%-polyacrylamide gel, and by comparison with low-molecular-mass standards was shown to be 8 kDa (results not shown). The radiolabelled 4-sulphatase found in the culture medium (approx. 25% of total) contained an additional 66 kDa polypeptide. Addition of NH_4Cl to the culture produced a 3-fold increase in radiolabelled 4-sulphatase in the culture medium. This contained only the 66 kDa polypeptide, which was shown to be unaffected by reduction (see Fig. 2*b*).

Radiolabelled 4-sulphatase polypeptides, identical in size and present in similar proportions, were isolated from fibroblasts incubated with either [^{35}S]methionine or [^{3}H]leucine and purified by using either the 4-S 4.1 or 4-S 5.2 monoclonal antibody (Fig. 2*c*). When lysates of radiolabelled cells or culture medium were applied to Tris-blocked Affi-Gel (Fig. 2*c*), only the large-molecular-mass proteins (> 200 kDa) bound; these are believed to be unrelated to 4-sulphatase. Similarly, neither the 66 kDa nor the 43 kDa polypeptides bound when radiolabelled fibroblast lysates were applied to an α-L-iduronidase-specific monoclonal-antibody Affi-Gel column (Clements *et al.*, 1989) (results not shown).

To examine the maturation sequence of 4-sulphatase, fibroblasts were pulse-labelled for 3 h with a radiolabelled amino acid and then subjected to a chase of up to 24 h. After a 3 h pulse, the 66 kDa polypeptide was the predominant radiolabelled intracellular species (Fig. 3*a*). The 120 kDa polypeptide observed at 3 h was not consistently detected, and its relationship to 4-sulphatase remains to be established. During the 3 h chase, the relative level of radioactivity in the 43 kDa polypeptide increased, and it became the major species by 24 h. A consistently observed trace of a 52 kDa polypeptide was apparent at 3 h, and the emergence of the 8 kDa polypeptide was detected by 24 h. These two polypeptides were detected by using both radiolabelled amino acids, but have been lost upon photographic reproduction (Fig. 3*a*). The culture medium showed an increase in incorporation of label into both the 66 kDa and 43 kDa polypeptides with increasing chase (Fig. 3*b*). The high-molecular-mass polypeptides (> 200 kDa) are believed to be unrelated to 4-sulphatase.

The half-life of 4-sulphatase in normal human fibroblasts in cell culture was determined after a pulse of 24 h and a prolonged chase. Assuming first-order kinetics for degradation, half-lives of 9, 15 (results not shown) and 17 days were determined for three different normal fibroblast cell lines. In all cases the radioactivity in the 43 kDa polypeptide decreased with time of incubation without the appearance of radiolabelled polypeptide degradation products (Fig. 4).

Synthesis and maturation of 4-sulphatase by MPS VI fibroblasts

The eight MPS VI patients studied were classified according to age of diagnosis and progression and severity of clinical symptoms (Table 1). Compared with normal fibroblasts, all MPS VI fibroblasts showed very low levels of incorporation of radiolabelled amino acid into 4-sulphatase, usually less than 3% of normal (Figs. 5 and 6). Consequently, four flasks of MPS VI
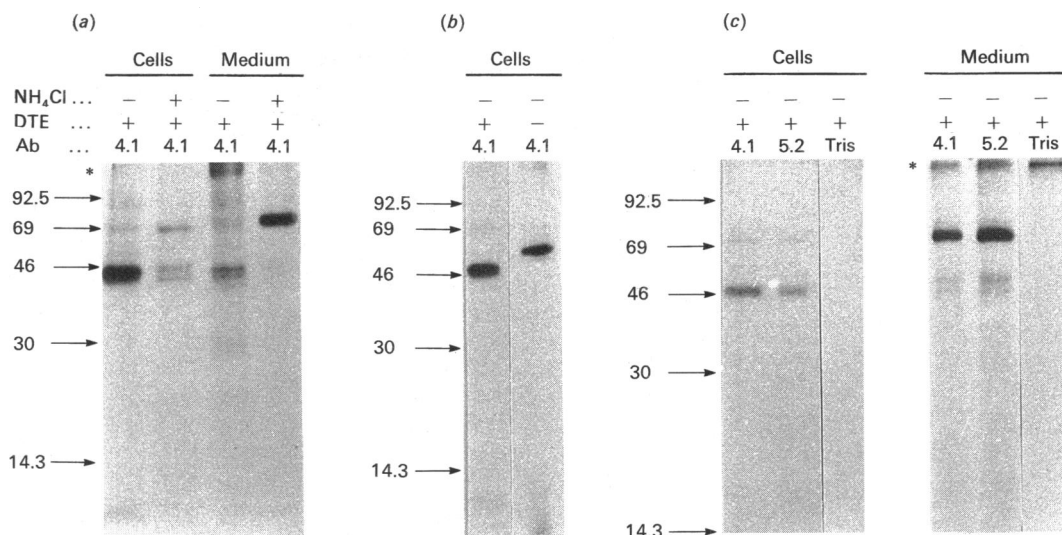
Fig. 2. SDS/polyacrylamide-gel electrophoresis and fluorography of 4-sulphatase immunopurified from normal fibroblasts

Fibroblasts (one flask per lane) were incubated for 24 h in the presence of [^{35}S]methionine, and 4-sulphatase was isolated from the cells and culture medium by immunopurification. (a) 4-Sulphatase isolated from cells and culture medium, incubated without (−) or with (+) 10 mM-NH$_4$Cl, and immunopurified on 4-S 4.1 Affi-Gel. (b) 4-Sulphatase isolated from cells on 4-S 4.1 Affi-Gel and run under reducing (+) or non-reducing (−) conditions. (c) 4-Sulphatase isolated from cells and culture medium and immunopurified on 4-S 4.1 Affi-Gel (4.1), 4-S 5.2 Affi-Gel (5.2) or Tris-blocked Affi-Gel (Tris). Ab indicates the type of antibody used. The asterisk * marks a contaminant believed to be unrelated to 4-sulphatase. Polyacrylamide-gel runs shown in (a), (b) and (c) were run separately, and some lanes in (b) and (c) have been re-positioned for ease of comparison. The positions of the molecular-mass standards (in kDa) are indicated by arrows. Abbreviation: DTE, dithioerythritol.
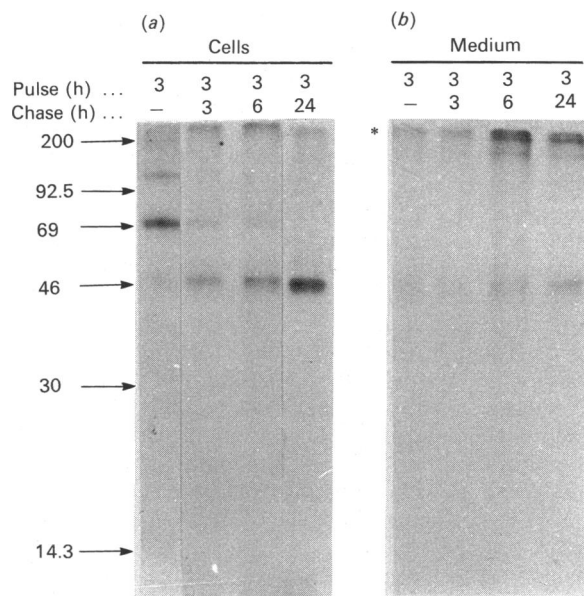

Fig. 3. SDS/polyacrylamide-gel electrophoresis and fluorography of 4-sulphatase isolated from pulse–chase-radiolabelled normal fibroblasts

Fibroblasts (one flask per lane) were incubated for 3 h in the presence of [^{35}S]methionine and harvested after a chase for up to 24 h as indicated. 4-Sulphatase was isolated from (a) the cell extracts or (b) the culture medium by immunopurification on 4-S 4.1 Affi-Gel. The asterisk * marks contaminants believed to be unrelated to 4-sulphatase. Polyacrylamide-gel runs shown in (a) and (b) were run on the same gel. The positions of the molecular-mass standards (in kDa) are indicated by arrows.

fibroblasts, compared with one flask of normal fibroblasts, were routinely used for the detection of radiolabelled 4-sulphatase.

The MPS VI patients have been further classified into groups

1 and 2 on the basis of the maturation of the 4-sulphatase synthesized by their fibroblasts in culture. Fibroblasts from the majority of patients (group 1, Table 2) incorporated radiolabel into both the 66 kDa and 43 kDa polypeptides. Fibroblasts from one patient (C.O.) showed a level of incorporation 2-fold higher than in fibroblasts from the other patients in this group and differed in the incorporation of label into 4-sulphatase in NH$_4$Cl-induced secretions. Fibroblasts from all MPS VI patients, except C.O., incorporated radiolabel into a 74 kDa polypeptide in NH$_4$Cl-induced secretions (Figs. 5c and 6c). Fibroblasts from C.O., however, like those from normal subjects, incorporated radiolabel into only the 66 kDa polypeptide in NH$_4$Cl-induced secretions (Table 2, Fig. 5c). The increased incorporation of radiolabel into 4-sulphatase achieved in normal fibroblasts by increasing the labelling time from 3 h to 24 h was not seen with fibroblasts from C.O. (Fig. 5a). The lower incorporation of radiolabel into 4-sulphatase detected with fibroblasts from other patients in this group prevented confident assessment of the relative levels of incorporation after 3 h and 24 h; however, a similar phenomenon appeared to occur (results not shown).

When fibroblasts from patients in group 1 (Table 2) were pulse-labelled for 3 h, the incorporation of radiolabel into the 66 kDa polypeptide, as detected in normal fibroblasts, was observed, although at a much decreased level. After a subsequent 6 h chase, however, the 66 kDa polypeptide remained as the major radiolabelled polypeptide. Only trace amounts of the 43 kDa polypeptide were detected (Fig. 5b). In normal fibroblasts the 43 kDa polypeptide is the predominant radiolabelled polypeptide after a 3 h pulse and 6 h chase (Fig. 3a). The incorporation of radiolabel into 4-sulphatase after a 3 h pulse and 6 h chase shown for patient M.F. (Fig. 5b) was the same as, and is representative of, that seen with fibroblasts from other patients in group 1 (Table 2).

Fibroblasts from two other MPS VI patients (J.W. and P.G.) (group 2, Table 2) incorporated radiolabel into a 52 kDa polypeptide both in cell lysates and also in the culture medium (Fig. 6). Fibroblasts from P.G. differed from those of J.W. (and
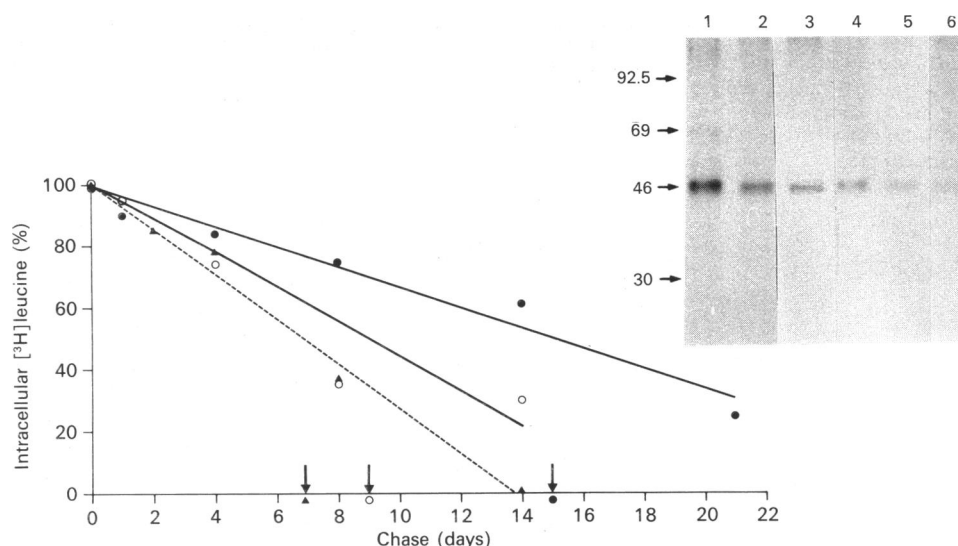
Fig. 4. Half-lives of 4-sulphatase from fibroblasts from two normal subjects and a MSD patient

Two normal fibroblast cell lines (○,●; one flask per time point) and one MSD cell line (N.B., ▲; one flask per time point) were incubated for 24 h with [³H]leucine and harvested after the chase time indicated. Radioactivity of the immunopurified 4-sulphatase was quantified by liquid-scintillation counting of the deoxycholate/trichloroacetic acid precipitates. Radioactivity over the chase period has been normalized relative to the amount of radioactivity in 4-sulphatase present at 24 h ($t = 0$). Apparent half-lives are indicated by arrows. The insert depicts a fluorogram of a normal (●) half-life determination. Lane 1 represents immunopurified 4-sulphatase after a 24 h pulse, and lanes 2, 3, 4, 5 and 6 after a chase of 1, 4, 8, 14 and 21 days respectively. The positions of the molecular-mass standards (in kDa) are indicated by arrows.

Table 1. Identifying data for MPS VI patients

Urinary dermatan sulphate was determined by the method of Hopwood & Harrison (1982). Residual 4-sulphatase activity was determined by using a radioactive trisaccharide, by the method of Hopwood *et al.* (1986). Abbreviations: n.a., not available; *n*, number of individuals.

Patient	Age at diagnosis	Clinical phenotype	Urinary dermatan sulphate (g/mol of creatinine)	Residual 4-sulphatase activity (pmol/min per mg of fibroblast protein)
J.W.	44 years	extremely mild	1	0.6
C.H.	25 years	mild	2	0.4
C.O.	18 years	mild	4	1.9
S.L.	4.5 years	moderate	n.a.	0.7
P.G.	2 years	severe	79	0.7
E.K.	1.5 years	severe	n.a.	0.6
S.B.	1 years	severe	n.a.	0.4
M.F.	7 days	severe	34	0.3
Control	< 2 months	normal	< 4*	11.8–39.2 ($n = 73$)
	2 months–2 years		< 2*	
	2–6 years		< 1*	
	> 6 years		< 0.7*	

* Control urinary glycosaminoglycan ranges ($n > 1000$) determined in the Department of Chemical Pathology, Adelaide Medical Centre for Women and Children.

the other MPS VI patients), in that incorporation of radiolabel into the 43 kDa polypeptide could not be detected. NH₄Cl-induced secretions of fibroblasts from J.W. and P.G. also contained the 74 kDa polypeptide which was seen with fibroblasts from group I (Fig. 6c).

Synthesis and maturation of 4-sulphatase by MSD fibroblasts

All three MSD fibroblast lines studied incorporated radiolabelled amino acids into the 66 kDa, 43 kDa and 8 kDa 4-sulphatase polypeptides (Fig. 7). The incorporation of radiolabel into 4-sulphatase in the cell lysates (Fig. 7) and culture medium (results not shown) after labelling for 24 h was decreased

compared with that incorporated into normal cell lines. The culture medium of the MSD fibroblasts also showed similar polypeptides to those observed in normal fibroblasts (results not shown). The stability of the 4-sulphatase 43 kDa polypeptide in fibroblasts from the MSD patient N.B. was examined after a 24 h pulse, followed by a prolonged chase, and the half-life was calculated as 7 days (Fig. 4).

DISCUSSION

The use of immobilized 4-sulphatase-specific monoclonal antibodies, combined with an extensive wash and elution protocol,

Table 2. Classification of MPS VI patients according to their pattern of incorporation of radiolabelled amino acids into 4-sulphatase polypeptides

4-Sulphatase was immunopurified from cell extracts or culture medium on 4-S 4.1 Affi-Gel, and the polypeptides were separated on SDS/polyacrylamide-gel electrophoresis and detected by fluorography.'Cells': fibroblasts from normal or MPS VI patients were cultured in the presence of radiolabelled amino acid. 'Medium': medium of fibroblasts cultured in the presence of 10 mM-NH_4Cl. 'Pulse': fibroblasts were cultured for 3 h or 24 h. Symbols: − indicates polypeptides clearly absent; + indicates polypeptides clearly present; ? indicates extremely faint bands suggestive that polypeptides may be present.

Patient group	Pulse (h)	4-Sulphatase polypeptides (kDa)...	Cells				Pulse (h)	Medium			
			74	66	52	43/8		74	66	52	43/8
Control	3		−	+	−	−	24	−	+	−	?
	24		−	+	+	+					
Group 1A											
M.F.	3		+	+	−	+	24	+	+	−	+
E.K.	3		−	+	−	+	24	+	+	−	+
S.B.	3		+	+	−	+	24	+	+	−	+
S.L.	3		−	+	−	+	24	+	+	−	+
C.H.	3		+	+	−	+	24	+	+	−	+
Group 1B											
C.O.	3		−	+	−	?	24	−	+	−	−
	24		−	+	−	+					
Group 2A											
J.W.	24		+	+	+	+	24	+	+	−	+
Group 2B											
P.G.	24		+	+	+	−	24	+	+	−	−
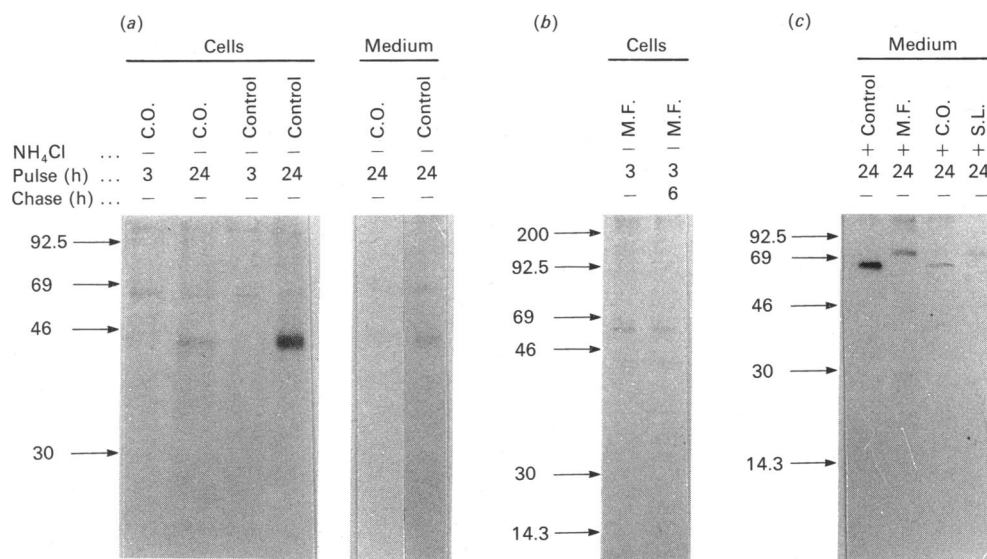
Fig. 5. SDS/polyacrylamide-gel electrophoresis and fluorography of 4-sulphatase isolated from fibroblasts of MPS VI patients (group 1, Table 2)

Fibroblasts were incubated for 3 h or 24 h in the presence of [^{3}H]leucine and harvested immediately, or after a chase of 6 h as indicated. 4-Sulphatase from the cells and culture medium was immunopurified on 4.S 4-1 Affi-Gel. (a) Immunopurified 4-sulphatase from cells and culture medium from a normal subject (one flask per lane) and a MPS VI patient C.O. (four flasks per lane). (b) Immunopurified 4-sulphatase prepared from a MPS VI cell line of patient M.F. (four flasks per lane). (c) Immunopurified 4-sulphatase from the medium of cells of a normal subject (one flask per lane) and MPS VI patients M.F., C.O. and S.L. (four flasks per lane) incubated in the presence of 10 mM-NH_4Cl. Polyacrylamide-gel runs shown in (a), (b) and (c) were run separately. The positions of the molecular-mass standards (in kDa) are indicated by arrows.

has enabled a study of 4-sulphatase biosynthesis and maturation in fibroblast cultures. Using these techniques we have shown that 4-sulphatase is synthesized as a 66 kDa polypeptide precursor, which is processed within 3 h to a 57 kDa polypeptide composed of two disulphide-linked polypeptides of 43 kDa and 8 kDa. The 66 kDa polypeptide was also detected in culture medium, and its secretion was stimulated by the presence of NH_4Cl. This description of 4-sulphatase synthesis is in part consistent with a

previous report of 4-sulphatase synthesis (Steckel et al., 1983), but differs significantly from those studies. Steckel et al. (1983) described the synthesis of two forms of 4-sulphatase, both having precursor polypeptides of 66 kDa and mature polypeptides of 57 kDa. One of the two 57 kDa polypeptides, form I, is composed of disulphide-linked polypeptides of 47 kDa and 11 kDa or 14.5 kDa, and the other, form II, is composed of polypeptides of 40 kDa and 31 kDa. Our inability to detect form II with two
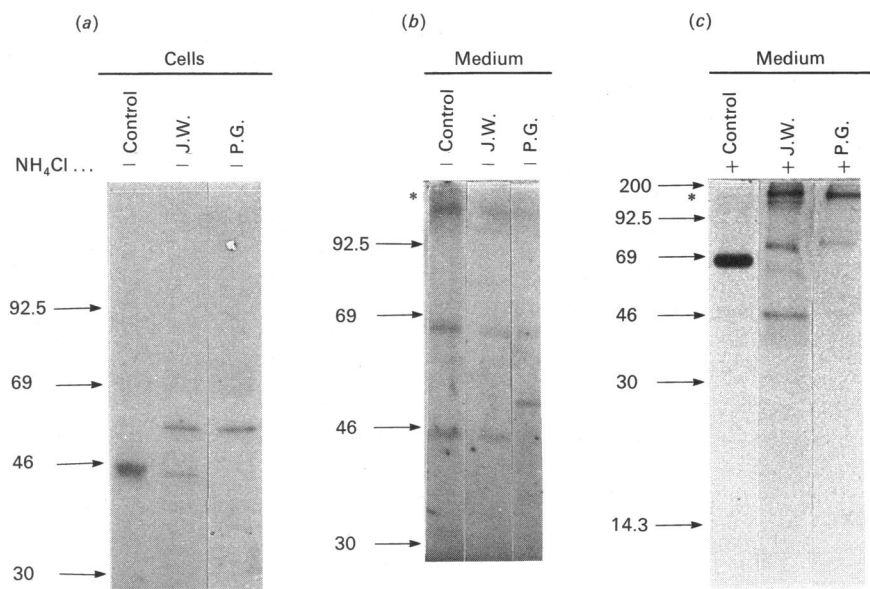
Fig. 6. SDS/polyacrylamide-gel electrophoresis and fluorography of 4-sulphatase immunopurified from fibroblasts of MPS VI patients J.W. and P.G. (group 2, Table 2)

Fibroblasts from MPS VI patients (four flasks per lane) were incubated for 24 h in the presence at [³⁵S]methionine. 4-Sulphatase was immunopurified on 4-S 4.1 Affi-Gel from (*a*) cells, (*b*) culture medium and (*c*) cells incubated with 10 mM-NH₄Cl. The asterisk * marks contaminants believed to be unrelated to 4-sulphatase. Immunopurified 4-sulphatase shown in (*a*) and (*b*) was electrophoresed on 7.5% acrylamide and in (*c*) on 12.0% acrylamide. Polyacrylamide-gel runs shown in (*a*), (*b*) and (*c*) were run separately, and some lanes in (*a*), (*b*) and (*c*) have been re-positioned for ease of comparison. The positions of the molecular-mass standards (in kDa) are indicated by arrows.
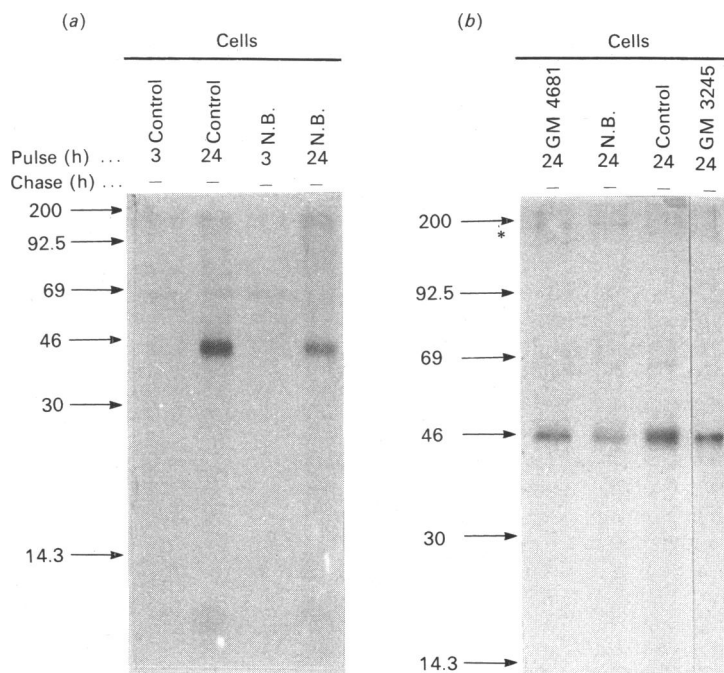

Fig. 7. SDS/polyacrylamide-gel electrophoresis and fluorography of 4-sulphatase immunopurified from fibroblasts of MSD patients

Fibroblasts from a normal subject (one flask per lane) and three MSD cell lines (N.B., GM 3245 and GM 4681) (two flasks per lane) were incubated for the times indicated in the presence of radiolabelled amino acids. 4-Sulphatase from the cells was immunopurified on 4-S 4.1 Affi-Gel. (*a*) Immunopurified 4-sulphatase prepared from a normal or a MSD cell line (N.B.) incubated for the times indicated in the presence of [³H]leucine. (*b*) Immunopurified 4-sulphatase prepared from a normal subject or from MSD cell lines incubated for the times indicated in the presence of [³⁵S]methionine. The asterisk * marks a contaminant believed to be unrelated to 4-sulphatase. Polyacrylamide-gel runs shown in (*a*) and (*b*) were run separately, and some lanes in (*b*) have been re-positioned for ease of comparison. Positions of molecular-mass standards (in kDa) are indicated by arrows.

distinct monoclonal antibodies (4S 4.1 and 4S 5.2) that recognize separate epitopes on 4-sulphatase, and the high recovery from the monoclonal-antibody columns, suggest that the purported form II may be a contaminant protein arising from non-specific antibodies in the antisera used by Steckel et al. (1983). This is further supported by the immunoreactivity of a 4-sulphatase-specific rabbit polyclonal antibody recently prepared in our laboratory which reacts with the 57 kDa, 43 kDa and 8 kDa polypeptides of 4-sulphatase, but does not interact with 40 kDa and 30 kDa polypeptides in our preparations (Brooks et al., 1990). The specificity of antibody preparations used in the isolation of 4-sulphatase assumes a greater significance in studies of the synthesis and maturation of 4-sulphatase in MPS VI fibroblasts in cell culture. Steckel et al. (1983) suggested that the 4-sulphatase precursor was synthesized by fibroblasts from MPS VI patients at a normal rate, but that the form (form I) generating active enzyme was unstable. An alternative explanation, and one compatible with our results, is that the precursor they observed was the contaminant (form II) only, and that the synthesis of form I (4-sulphatase), both precursor and mature forms, was, as we observed, greatly decreased.

In all MPS VI fibroblasts that we have examined, and possibly those reported by Steckel et al. (1983), the deficiency of 4-sulphatase catalytic activity appears to arise from a mutation affecting the synthesis of the enzyme. It should be recognized, however, that the apparent decrease in 4-sulphatase synthesis could arise if the mutation caused the loss or masking of the monoclonal-antibody epitope on 4-sulphatase or if the mutation caused the enzyme to be degraded very soon after synthesis. Characteristics of the maturation of the 4-sulphatase synthesized by fibroblasts from MPS VI patients facilitated the division of our MPS VI patients into two groups. Such a classification relies on the detection of low levels of incorporation of radiolabel and must recognize the probability that a mutation may cause multiple effects on enzyme synthesis, stability and maturation. Furthermore, these classifications may be further complicated since a number of patients are likely to be double heterozygotes and thus synthesize two different mutant 4-sulphatases.

Several features of 4-sulphatase maturation in fibroblasts from MPS VI patients suggest that the mutation leading to decreased synthesis also caused decreased enzyme stability, decreased efficiency of maturation and possible increased intracellular transport time, and suggest that the correct and efficient post-translational modification, transport and lysosomal targeting may be very sensitive to subtle changes in the secondary and tertiary structure of 4-sulphatase which may result from mutation. The frequent observation of a 74 kDa polypeptide immunopurified from NH_4Cl-induced secretions of fibroblasts from MPS VI patients may be a consequence of delayed intracellular transport and the resultant increased glycosylation of the 4-sulphatase enzyme. The 52 kDa polypeptide seen in the cultured fibroblasts of two MPS VI patients (group 2) is similar in size to the intermediate observed in 4-sulphatase maturation in normal fibroblasts, and may reflect a slowing or block (in the case of P.G.) of subsequent cleavage to the mature 43 kDa and 8 kDa polypeptides. Since early proteolytic events (i.e. for 4-sulphatase, 66 kDa cleavage to 52 kDa) appear to occur in pre-lysosomal compartments (Gieselmann et al., 1983; Oude Elferink et al., 1986; Gabel & Foster, 1987), this may indicate a disruption or block of subsequent transport to the lysosome.

From studies of cultured fibroblasts, we conclude that the synthesis of 4-sulphatase in MPS VI patients was severely decreased, but in MSD patients was close to the normal range. Although the mutations producing decreased 4-sulphatase synthesis in MPS VI patients also affected the stability, intracellular trafficking and lysosomal targetting of the enzyme, we have been unable to correlate any of these effects with clinical severity. Changes in 4-sulphatase synthesis in MSD patients are much more subtle. Although there is some evidence of a decrease in synthesis of 4-sulphatase, the processes of targetting, maturation and lysosomal stability appear close to normal and presumably reflect the results of mutation in a second protein, 'the multiple sulphatase factor' (Waheed et al., 1982). It is apparent that the severe clinical characteristics of multiple sulphatase deficiency can result from the cumulative, though possibly individually mild, deficiency of all sulphatases.

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